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Original Article

The Combination of LPS and Melatonin Induces M2 Macrophage Apoptosis to Prevent Lung Cancer

Fan Gao[1#], Yukun Lin[1#], Mengdi Zhang[1#], Yuju Niu[1], Luyao Sun[1], Wenwen Li[1], Haojie Xia[1], Haihong Lin[1], Zhenzhen Guo[1*] and Gangjun Du[1,2*]

[1]*Institute of Pharmacy, Pharmaceutical College of Henan University, Kaifeng, Henan, China;* [2]*School of Pharmacy and Chemical Engineering, Zhengzhou University of Industry Technology, Xinzheng, Henan, China*

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Abstract

Background and objectives: Lasting LPS stimulation changes macrophages toward the M2 phenotype therefore resulting in immunodepression. Melatonin can improve sleep, adjust the time difference, regulate immunity, and anti-tumor. This study is to observe whether melatonin can induce M2 macrophage apoptosis to reverse lasting LPS-induced immunodepression for lung cancer prevention and explore the possible mechanism.

Methods: The effects of LPS alone or in combination with melatonin on macrophage phenotypes were assessed by surface markers, morphological changes, cytokines, autophagy, and autophagic efflux. The anti-cancer effect was evaluated in the lung carcinogenic model and lung cancer allograft model. Melatonin-related targets and pathways were predicted by network pharmacology.

Results: Single LPS stimulation polarized macrophages to M1 phenotype, whereas LPS stimulation lasting for 7d polarized macrophages to M2 phenotype. However, combination treatment of lasting LPS and 10 μM melatonin inhibited the polarization of macrophages towards an M2-like phenotype and exerted a continuous antitumor effect. In the urethane-induced lung carcinoma model, long-lasting LPS administration (>4 times) facilitated macrophage polarization toward the M2 phenotype and promoted lung carcinogenesis, which was abrogated by macrophage depletion, while melatonin alone or in combination with lasting LPS could decrease M2-like macrophages and prevented carcinogenesis. In the Lewis lung cancer allograft model, melatonin decreased M2 macrophages and promoted the tumor-suppressing effect of short-term LPS administration (<4 times). Network pharmacology indicated that melatonin regulates macrophages by targeting the multi-protein network.

Conclusions: Melatonin as a key maintainer of macrophage phenotype can induce LPS-stimulated M2 macrophage apoptosis to reverse system immunodepression for lung cancer prevention.

Keywords: Melatonin; Macrophage phenotype; LPS, Carcinogenesis; Clinical application.

Abbreviations: DAVID, Database for Annotation, Visualization and Integrated Discovery; FBS, fetal bovine serum; GEO, Gene Expression Omnibus; GO, Gene Ontology; ICB, immune checkpoint blockade; KEGG, Kyoto Encyclopedia of Genes and Genomes; LEC, liposome-encapsulated clodronate; LLC, Lewis lung carcinoma; LPS, Lipopolysaccharide; PDB, the protein data bank; PPIs, The protein-protein interactions; TIMs, Tumor-infiltrated macrophages.

[*]**Correspondence to:** Gangjun Du, School of Pharmacy and Chemical Engineering, Zhengzhou University of Industry Technology, Xinzheng 451150, Henan, China. ORCID: https://orcid.org/0000-0003-3419-2218. Tel: +86-0371-23880680, Fax: +86-0371-23880680, E-mail, 10200029@vip.henu.edu.cn; Zhenzhen Guo, Institute of Pharmacy, Pharmaceutical College of Henan University, Jinming District, Kaifeng 475004, Henan, China. ORCID: https://orcid.org/0000-0002-3228-3741. Tel: +86-0371-23880680, Fax: +86-0371-23880680, E-mail: 1070417690@qq.com

[#]These authors contributed equally to this work.

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Introduction

Cancer is one of the leading causes of human disease-related death, and much attention regarding cancer treatment has focused on targeting and killing the tumor itself, *e.g.*, radiation, chemotherapy, and targeted therapy.[1–5] Recently, cancer immunotherapy has become one of the most promising therapeutic pillars in improving patient survival.[6,7] Unlike other cancer treatments, can-

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cer immunotherapy activates the host's immune system or relieves "immune exhaustion" in the tumor environment to eliminate cancer cells.[8] The data analysis of front-line anti-CTLA4 therapy exhibited that the long-term survival in 20% of terminal metastatic melanoma patients reached an inspiring high value that had never appeared before in other therapy, indicating an exciting success in cancer immunotherapy that can provide hope of becoming "super-survivors" to incurable cancer patients.[9,10] Nevertheless, response rates with the most promising immunotherapy, such as PD-1 inhibitors, which as the representative of immune-checkpoint-blockade (ICB) could mediate the regeneration of depletion T cells, only exhibit a modest 20% overall survival benefits in many solid tumors, besides, CAR-T cell therapy was reported to result in cerebral edema and cytokine release storms.[11] In addition, a more recent clinical report showed that PD-1 inhibitors could speed up tumor growth and promote tumor hyperprogression in 9% of cancer patients, indicating a need to optimize cancer immunotherapeutic approaches. Current immunotherapies mainly activate tumor-infiltrating T lymphocytes and natural killer cells without regard to environmental changes that can cause various dysfunctional states of T cells, for example, senescence, tolerance, exhaustion, and energy.[12] In fact, antitumor T cell immunotherapies originally exhibited responses but subsequently became resistant during prolonged antigen exposure due to "immune exhaustion" induced by the immunosuppressive microenvironment that is shaped by tumor-associated inflammatory cells.[13] Obviously, effective tumor immune rejection relies not only on antigen exposure-induced adaptive immune responses but also on the innate immune surveillance-regulated microenvironment.[14]

Tumor-infiltrated macrophages (TIMs), M2-like macrophages, are major inflammatory cells infiltrating the tumor microenvironment and are responsible for the immunosuppressive microenvironment and tumor progression.[15] If macrophage-mediated phagocytosis is suppressed, it will give rise to immune evasion of tumors.[16] In the clinic, TIMs are positively related to high-tumor grade and certain poor prognosis in various cancers. In mouse cancer models, TIM depletion or reeducation can reverse their tumor-promoting functions.[17,18] In addition, innate macrophages also play important roles in the intratumor infiltration of CD8 cytotoxic T cells and the establishment of the long-lived memory lymphocytes. Therefore, targeting TIMs to reawaken innate immunity has emerged as a new cancer immunotherapy strategy.[19] However, up until now, anti-TIM therapies are not only at risk of systemic toxicities but also may be adapted and escaped by macrophage pool rapidly.[19] As is known to all, activated macrophages can polarize into antitumor M1 and protumor M2 phenotypes.[20] LPS can polarize macrophages toward the M1 phenotype, but lasting stimulation results in immune tolerance.[21] Melatonin, a neurohormone, secreted by the pineal gland and can improve sleep, adjust the time difference, regulate immunity, and anti-tumor.[22] This study is to investigate whether the combination of LPS and melatonin can induce M2 macrophage apoptosis to cause a lasting lung cancer-preventing effect.

Materials and methods

The preparation of the "Materials and Methods" section followed the Animal Research: In Vivo Experiments (ARRIVE) guidelines (see Supplementary File 1),

Materials

Melatonin (purity > 98%) was bought from Lianshuo Biotechnology Co., Ltd. (Shanghai, China). Urethane, LPS, and filipin were purchased from Sigma Chemical Co (St. Louis, MO, USA). Anti-F4/80-coated beads were purchased from BioCep (Israel). Antibodies used included anti-mouse-arginase, anti-mouse-iNOS, anti-mouse-F4/80, anti-mouse-CD8, anti-mouse-Foxp3, Cy3-conjugated anti-mouse CD86, PE-conjugated anti-mouse CD163, and FITC-conjugated goat anti-mouse IgG were obtained from BD Pharmingen or Proteintech (Shanghai China). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG polyclonal antibody and peroxidase substrate DAB (3, 3′-diaminobenzidine) were obtained from Nichirei Bioscience (Tokyo, Japan).WP1066, DCFH-DA, MβCD, and LY294002 were bought from Beyotime (Shanghai China). ELISA kits for a mouse (IFN-γ, TGF-β1, IL-2, IL-10, and ROS) and Annexin V-FITC Apoptosis kit were obtained from R&D Systems. L-arginine and nitric oxide assay kits were obtained from Nanjing Jiancheng Bioengineering Institute. Standard rodent chow was purchased from Henan Provincial Medical Laboratory Animal Center (Zhengzhou, China), License No. SCXK (YU) 2015-0005, Certificate No. 41000100002406. Liposome-encapsulated clodronate (LEC) was prepared as described previously.[23]

Cell culture and assay

Raw264.7 macrophages and the Lewis lung carcinoma (LLC) cells were from ATCC, purchased from the Chinese Academy of Sciences, and grown in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Raw264.7 macrophages were seeded in 24-well plates and stimulated with 10 ng/mL LPS or 10 ng/mL IL-10 for 24 h to obtain M1-like (M1) and M2-like (M2) macrophages. To collect cell-conditioned medium, M1-like or M2-like cells were cultured for 24 h in the medium without serum, centrifugated, and filtered to collect the supernatant as M1 or M2 cell-conditioned medium (M1-CM and M2-CM, respectively). The supernatant levels of IFN-γ, IL-10, TNF-α, NO, TGF-β1 and PD-L1 were tested by ELISA kits.[24]

For analysis of proliferation, LLC cells were seeded in a 96-well plate (1×10^5 cells/mL) and treated with M1, M2, or M0 cell-conditioned medium for 48 h, M1-like or M2-like cells were given LPS or single melatonin or in combination for 7 d (changing the medium every 2 days), and living cells were examined by MTT assay.[23] For the morphological assessment, cellular morphology was measured by the Laser holographic cell imaging and automatic algorithm software.[24] For phagocytic ability assessment, neutral red phagocytosis was detected. For autophagy analysis, the cells were stained by utilizing PE-conjugated anti-LC3-B or anti-p62 antibodies. In apoptosis analysis, the binding of ANXV-FITC with phosphatidylserine was detected. In lipid raft detection, cells were stained by utilizing filipin (0.05 mg/mL) for plasma membrane cholesterol, respectively according to conventional methods. In ROS measurement, the fluorescence spectrophotometer was utilized to measure the intracellular fluorescence of DCFH-DA (F4600, Hitachi, Japan).

Western blot analysis

M1-like and M2-like macrophages were given LPS or single melatonin or in combination under the condition that adding or not of MβCD (a kind of lipid raft inhibitor), WP1066 (a kind of selective JAK2/STAT3 inhibitor) for 48 h. The expression levels of PD-1, PD-L1, JAK2, and pStat3 were measured by Western blot analysis. Following the manufacturer's instructions (Pierce, Rockford, IL) and using the ImageJ software (NIH, Bethesda, MD, USA), band

density was quantified, meanwhile, normalized to the corresponding control group.

Animals

Female ICR ten-week-old mice were purchased from Henan Provincial Medical Laboratory Animal Center and were randomly assigned by weight. Animals were fed standard fodder and water and were raised in individual cages with timed light. All procedures were allowed by the Animal Experimentation Ethics Committee of Henan University (permission number: HUSAM 2016-288), in addition, all operations were conducted according to the Guide for the Care and Use of Laboratory Animals and the Regulation of the Animal Protection Committee strictly. Animals were executed via carbon dioxide overdose based on experimental needs.

LLC cell immune clearance

Immune clearance of LLC cells was evaluated by calcein-release assay.[23] Briefly, macrophages (F4/80+) were extracted and purified from the ICR mouse spleens using the MACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany), then, treated with LPS (10 ng/mL) in the presence of M1-like or M2-like cell-conditioned medium for 24 h and collected as attacking cells. At 37 °C, LC cells were treated with mitomycin C and labeled by 10 μM calcein-AM as target cells and were seeded with macrophages at 100:1, 50:1, and 25:1 (macrophages: LLC cells) ratios in a 96-well plate for 6 h. The supernatants of each well were measured by the Synergy2 multimode microplate reader (BIO-TEK) in a new 96-well plate. The number of detergent-released LLC cells was taken as maximum release, and the number of LLC cells incubated without the condition of macrophages was taken as the spontaneous release. (n = 5).

The calculating formula for immune clearance rate is as follows:

$$\text{Computational formula of immune clearance rate} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100\%$$

To explore how the different macrophage immunophenotypes influence macrophages, macrophages (F4/80+) from the ICR mouse spleens cells were seeded in the lower culture chamber (5×10^6 cells/mL) and were stimulated with LPS and M1-like or M2-like cells were seeded in the upper culture chamber (2×10^6 cells/mL) with the conditions of adding or not of anti-IL-10, anti-TGF-β1, MβCD, WP1066. Co-culture at 37°C for 24 hours, after that, the supernatant in the chamber was centrifugated for PD-L1 assay, and the filter inserts were stained by 5 mg/mL DAPI after removing the adherent cells in the upper chamber for analyzing the cell migration. The macrophages in the lower compartment were harvested to conduct a surface PD-1 receptor assay by using a PE-conjugated anti-PD-1 antibody.

Cytolytic assay

CFSE-7AAD staining assay was utilized to evaluate the cytolytic activity of LLC cells. Briefly, LLC cells were incubated with CFSE-labeled M0, M1-like, or M2-like cells at 20:1 and 10:1 (M0, M1, or M2 cells: LLC cells) ratios for 6 h. After adding the 7AAD, cell suspensions were stood for 15 min on ice. Proportion of 7AAD + cells in the CFSE + cells were detected.

Urethane-induced lung carcinogenesis model

Urethane (600 mg/kg), single administration or in combination with LEC (4 mg/mouse) was intraperitoneally injected into mice once per week for 8 weeks, according to our previous protocol.[25] Following the first time of urethane injection, mice were given melatonin (20 mg/kg/day) by gastric infusion once per day or LPS (1 mg/kg/day) by tail vein injection once a week single administration or in combination for 12 weeks. At thirteen weeks after the first injection of urethane, orbital venous blood was harvested for expression analysis of IFN-γ, ROS, IL-2, TGF-β1, TNF-α, and IL-10 by utilizing an ELISA kit. The mice were executed, the cell-free alveolar fluid was harvested and centrifugated for cytokine assay (IFN-γ, ROS, IL-2, TGF-β1, TNF-α, and IL-10), then, the number of total cells was counted by hanging the separated cells in 0.9% sterile saline. Macrophages in the suspensions were enriched by anti-F4/80-coated beads and purified by the magnetic cell sorting, after that, these macrophages were stained by FITC-conjugated anti-mouse CD86 and CD163 for the test of immunophenotypes.

Spleen macrophages (F4/80+) were separated by utilizing an autoMACS separation system for assays of surface PD-1 and intracellular IL-12 and iNOS.

The mean number of nodules in the lung of each mouse was figured out. A part of every lung was soaked in the 10% buffered formalin or routinely embedded in paraffin to be preserved. Sections of the lung were stained and analyzed by immunohistochemistry and immunofluorescence.[26] The slides were co-incubated with primary antibodies (anti-PD1, anti-iNOS, and anti-CD31) overnight, then, incubated with a second antibody for half an hour. The final scores of immunohistochemical and immunofluorescence were synthetically calculated by the intensity score and proportion score by excluding the primary antibody and IgG matched serum, respectively, as positive and negative controls.

And the lung vascular integrity was detected by the Evans blue dye extra barrier experiment.[27]

Tumor allograft model

LLC cells were utilized to establish the model of tumor allograft. Two hundred microliters of saline containing 1×10^6 LLC cells and 2×10^6 LPS-induced M1-like cells or IL-10-induced M2-like cells were injected hypodermically into the axilla of mice to establish tumor allografts. The day after tumor cells inoculation, mice were given melatonin (20 mg/kg) once per day through gastric infusion and LPS (1 mg/kg) or LEC (4 mg/mouse) through tail vein injection once a week or in combination for 3 weeks. The lengths and widths per tumor were measured twice a week by using calipers and the sizes per tumor were calculated as width^2 × length/2. On the twenty-second day after tumor cells inoculation, the orbital venous blood of each mouse was harvested for serum assays of IFN-γ, ROS, IL-2, TGF-β1, TNF-α, and IL-10. The mice were sacrificed, and the tumors were take-out and weighed, peritoneal macrophages were enriched by anti-F4/80-coated beads and purified by the magnetic cell sorting, and macrophage immunophenotypes were analyzed. Spleen macrophages were separated by using the autoMACS separation system for assays of surface PD-1 and intracellular IL-12 and iNOS. Moreover, the vascular integrity of the tumor was analyzed by the Evans blue dye extra barrier experiment.[24]

In the immune rechallenge study, 40 mice were injected hypodermically in the midpoint position of tails with 50 μl LLC cells suspension (1×10^7 /mL). The day after injection of cells, these

mice were given melatonin (20 mg/kg) through gastric infusion once per day and LPS (1 mg/kg) or LEC (4 mg/mouse) single or in combination through tail vein injection once a week for 3 weeks. Fifteen days after injection of the tumor, the primary tumor was removed by cutting off the tumor-bearing tail, and these mice were rechallenged by hypodermic inject 0.2 mL LLC cells suspension liquid with the concentration of 5×10^6 /mL in the axillae, while 10 normal mice were challenged with identical LLC cells. The volume of the tumor was detected by a caliper. At thirty-six days, the same examination above was performed on these mice.

The mechanism of melatonin on macrophages regulation

GSE5099 were downloaded from GEO online database, M1 macrophage related up-regulated and down-regulated genes (macrophages at day 7 vs IFN-γ and LPS treated macrophages) were exhibited by the tool of GEO2R, and the human structures of these differential proteins were downloaded from the protein data bank (PDB) for molecular docking. The chemical structure of melatonin was downloaded from PubChem, and the docking was performed by systemsDock. These potential targets have the condition of a docking score that is greater than 5. GO and KEGG analyses were performed by using the DAVID database, meanwhile, the protein-protein interactions (PPIs) of potential targets were downloaded from the String database, and hub genes were selected by Cytoscape.

Statistical analyses

The data were statistically analyzed using GraphPad Prism, Version 5.0 (San Diego, CA, USA) and are presented as the mean ± SD. The sample size of the experiments was decided by experimental need, and the experiment was repeated three times. The differences between the two groups were evaluated using a t-test. A P value of less than 0.05 was considered statistically significant.

Results

M2 macrophages had an opposing effect on LLC cells compared to that of M1 macrophages

It was reported that cancer-associated macrophages have M2-like characteristics and exert tumor-promoting actions, unlike M1-like macrophages, which show antitumor functions.[28] To explore these properties, we induced Raw264.7 macrophages to polarize into M1-like cells by short-term stimulation of single LPS and M2-like cells by lasting stimulation of single LPS. As expectedly, the expression of surface CD86 in M1-like cells is high, and the expression of surface CD163 in M2-like cells is high (Fig. 1a, b), and the cell distribution also indicated their morphological changes of them (Fig. 1c, d). In neutral red phagocytosis, M1 cells are not significantly different from M2 cells (Fig. 1e); however, M2-like cells had a decline in levels of ROS (Fig. 1f), IFN-γ, TNF-α, NO (Fig. 1g) and an increased levels of PD-L1, IL-10, TGF-β1 (Fig. 1h). Simultaneously, M2-like cells had decreased autophagy and autophagic efflux indicated by the less LC3-B and the more p62 (Fig. 1i). In function, M1-like cells had stronger cytolytic activity on LLC cells than M2-like cells (Fig. 2a), M1 cell-conditioned medium (M1-CM) had also a stronger inhibitory effect on LLC cell proliferation (Fig. 2b) and resulted in more apoptotic rates (Fig. 2c) than M2 cell-conditioned medium (M2-CM). As there was no significant difference between the control medium and M0 condition medium in the MTT assay, and considering the purpose of this experiment is to investigate the effect of the combination of LPS and melatonin on macrophages to prevent lung cancer, M0 conditioned medium was no longer used as a negative control in subsequent experiments. In addition, the coculture of M1-like cells with NK cells decreased PD-1 expression of NK cells, which was promoted by that of M2-like cells, this efficacy could be aborted by pretreatment of MβCD, anti-TGF-β1 antibody, and anti-IL-10 antibody in M2-like cells but not in M1-like cells, indicating a lipid rafts/cytokines dependent effect of M2 macrophages on NK cell function (Fig. 2d). Consistent with this result, M1-CM promoted immune clearance of LLC cells by NK cells (Fig. 2e), which was prevented by M2-CM.

M2 macrophage function is associated with lipid rafts and JAK2/STAT3 signal

Large amounts of signal transduction related to cell survival, migration, and cell adhesion are dependent on lipid rafts.[29] Based on the above results, we detected differences between M1-like macrophages and M2-like macrophages in lipid rafts and signal activation. The results showed that M2-like macrophages had more lipid rafts compared to M1 cells., as indicated by membrane cholesterol (Fig. 2f). Simultaneously, M2-like cells produced more JAK2/pSTAT3 activation and PD-L1 than M1-like cells (Fig. 2g), WP1066, a JAK2/STAT3 signal blockade, could decrease PD-L1 production in M2-like cells (Fig. 2h), indicating the relevance of M2 macrophage function to lipid rafts and JAK2/STAT3 signal.

Melatonin decreases M2 macrophages to reverse the macrophage tolerance of LPS

Macrophages may be tolerant to LPS, an effect that lasts for a period of time.[30] To explore how macrophages become tolerant to LPS, we observed the effects of melatonin and LPS on macrophage polarization. As expected, lasting LPS treatment led to a transformation of macrophage immunophenotype from M1 toward M2 immunophenotype indicated by surface decreased CD86 and increased CD163 expression (Fig. 1a, b), this result was further confirmed by morphological changes (Fig. 1c, d). At the dose of 10 μM, Melatonin was not found to affect cell viability, however, could decrease M2 macrophage under the condition of repeated LPS treatment (Fig. 1a–d). Simultaneously, melatonin could promote a function shift of macrophages from the M2 to M1, such as, increased ROS (Fig. 1f), enhanced autophagy and autophagic efflux (Fig. 1i), and decreased lipid rafts and JAK2/STAT3 activation (Fig. 2f, g). The inhibitory effect of melatonin on JAK2/STAT3 activation and PD-L1 production was attenuated or aborted by WP1066 (Fig. 2h).

Melatonin inhibits macrophage M2 phenotype to reverse LPS-mediated carcinogenesis in a urethane-induced lung cancer model

To illustrate the influence of macrophages in carcinogenesis, we explored the immunophenotypes of macrophages in the mouse lung cancer model induced by urethane, this model is generally established to investigate the basic biology of lung cancer and to find a new intervention strategy for lung cancer. In our study,

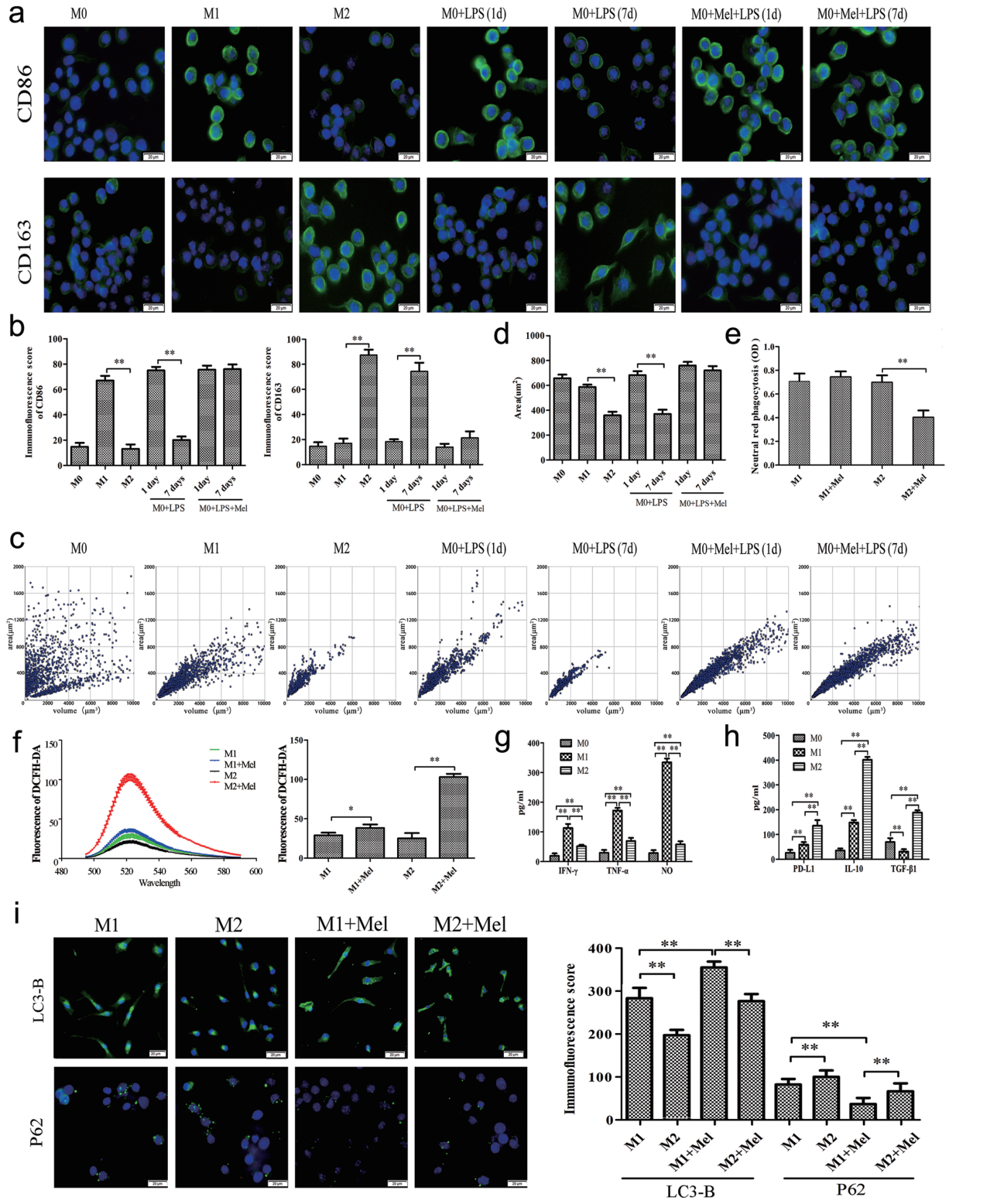

Fig. 1. There is a difference between M1-like and M2-like macrophages. M1-like and M2-like macrophages were induced respectively by LPS and IL-10 from Raw264.7 macrophages. (a) and (b) Macrophages CD86 and CD163 expression (n = 5, 40×). (c) and (d) Morphological changes of macrophages (n = 5, 20×). (e) Macrophages' phagocytosis detected by neutral red (n = 5, 40×). and (f) ROS detected by DCFH-DA (n = 5). (g) Supernatant levels of IFN-γ, IL-2 and NO in cell culture (n = 5). (h) Supernatant levels of PD-L1, IL-10 and TGF-β1 in cell culture (n = 5). (i) Cell autophagy as indicated by LC3-B and P62 staining (n = 5, 40×). **P* < 0.05, ***P* < 0.01. LPS, Lipopolysaccharide; Mel, Melatonin.

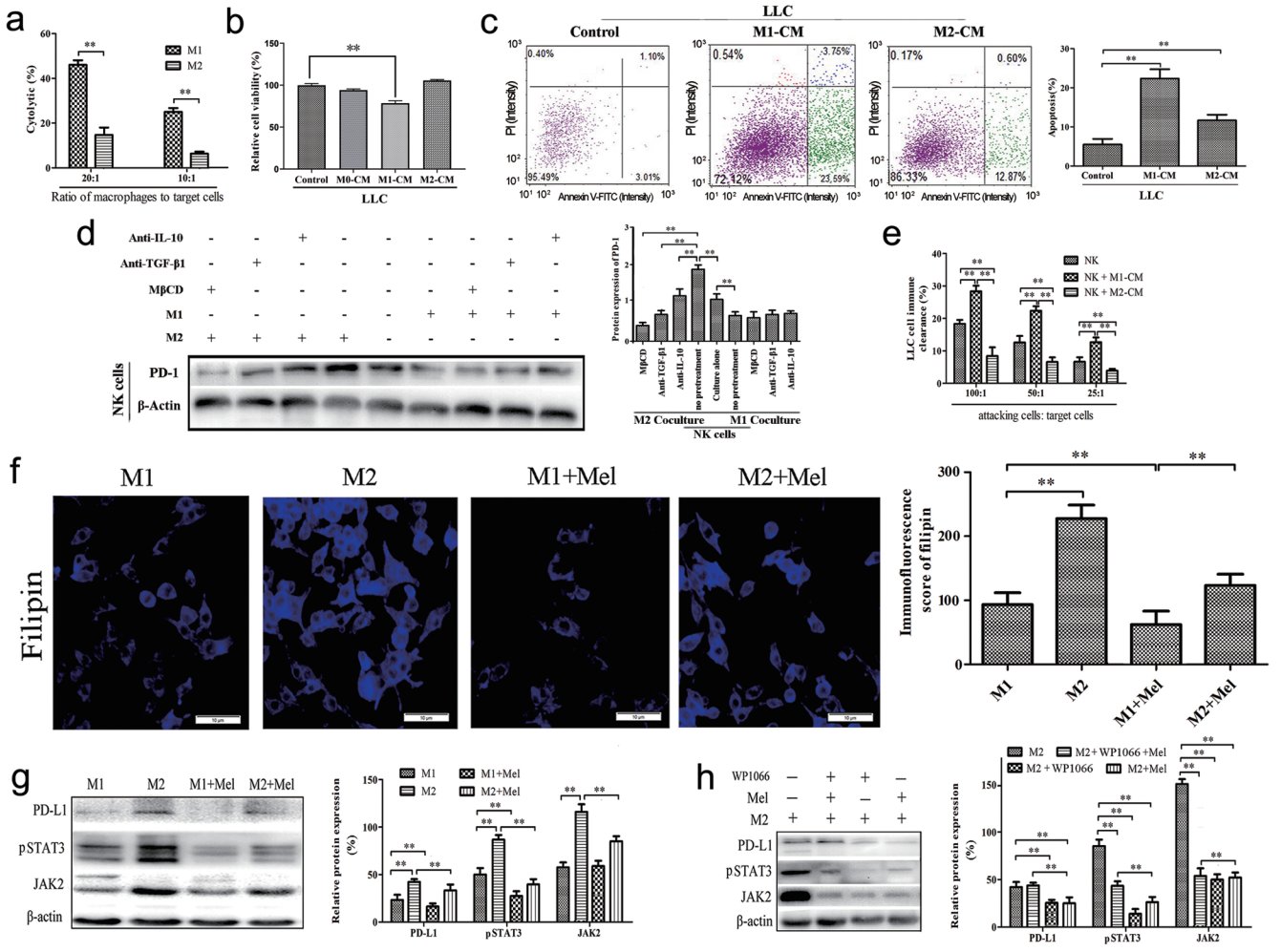

Fig. 2. M1-like and M2-like macrophages had different effects on LLC cells and NK cells. (a) Cytolytic activity (n = 5). (b) LLC cell viability detected by MTT assay (n = 5). (c) Cell apoptosis detected by the Annexin V-FITC apoptosis kit (n = 5). (d) PD-1 expression in NK cells (n = 5).). (e) NK cell immune clearance. (f) Lipid raft examined by filipin (n = 5). (g) and (H) Protein levels of PD-L1, pSTAT3 and JAK2 examined by Western blotting (n = 5). *P < 0.05, **P < 0.01. LLC, Lewis lung carcinoma; Mel, Melatonin.

the mice were given melatonin or LPS for twelve weeks. At thirteen weeks, lung cancer nodules were visible under the naked eye (Fig. 3a). The number of lung cancer nodules was 26.2±4.1 in the control group, the heterogeneity of tumor histology can be ignored (Fig. 3b). As was expected, there was a positive correlation between macrophage infiltration in alveolar cavities and lung cancer in the control group (Fig. 3c, f), these macrophages presented an M2 phenotype indicated by surface fewer CD86 and more CD163 expression (Fig. 3c–f). Consistent with this result, the levels of IFN-γ, IL-2, and TNF-α decreased, while the levels of IL-10, TGF-β1, and ROS increased in alveolar cavities and serum in control mice compared to those of normal mice (Fig. 3g, h), indicating immune tolerance and chronic injury. LPS alone did not prevent lung carcinogenesis but promoted these incidents (Fig. 3a, b), an increase in macrophage surface CD163 and the levels of ROS, TGF-β1, and IL-10 compared to those of control mice (Fig. 3c–f), indicating M2 polarization. Both melatonin and macrophage depletion mediated by LEC inhibited lung carcinogenesis (Fig. 3a, b), with the increase of a proportion of macrophage surface CD86 to CD163 and Th1 cytokines (IFN-γ and IL-2) to Th2 cytokines (IL-10 and TGF-β1) compared to those of control mice (Fig. 3c–f), indicating immune surveillance recovery. Importantly, the combination treatment of melatonin and LPS reversed the effect of lung carcinogenesis-promoting of LPS (Fig. 3a, b), accompanied by a reduction of the M2 macrophages indicated by macrophage surface CD86 and CD163 expression and the levels of IFN-γ, IL-2, TNF-α, IL-10, TGF-β1 and ROS (Fig. 3c–f).

Natural killer (NK) cells play a significant role in the antitumor immunity, to determine whether macrophages are involved in NK cells-associated anti-tumor function, we examined the percentage of spleen PD-1$^+$ NK cells and memory NK cells (NKG2C$^+$ NKG2A$^-$). The result showed that there was an obvious rise in the proportion of spleen PD-1$^+$ NK cells and a significant reduction in the proportion of spleen memory NK cells in control mice compared to those of normal mice (Fig. 4a, b), these trends were promoted by LPS but were attenuated by melatonin alone or in combination with LPS and macrophage depletion induced by LEC (Fig. 4a, b), which was consistent with the changes of macrophage phenotypes, suggesting an inhibitory effect of M2 macrophages on NK cells. Consistent with this result, lung tissues had more infiltration of

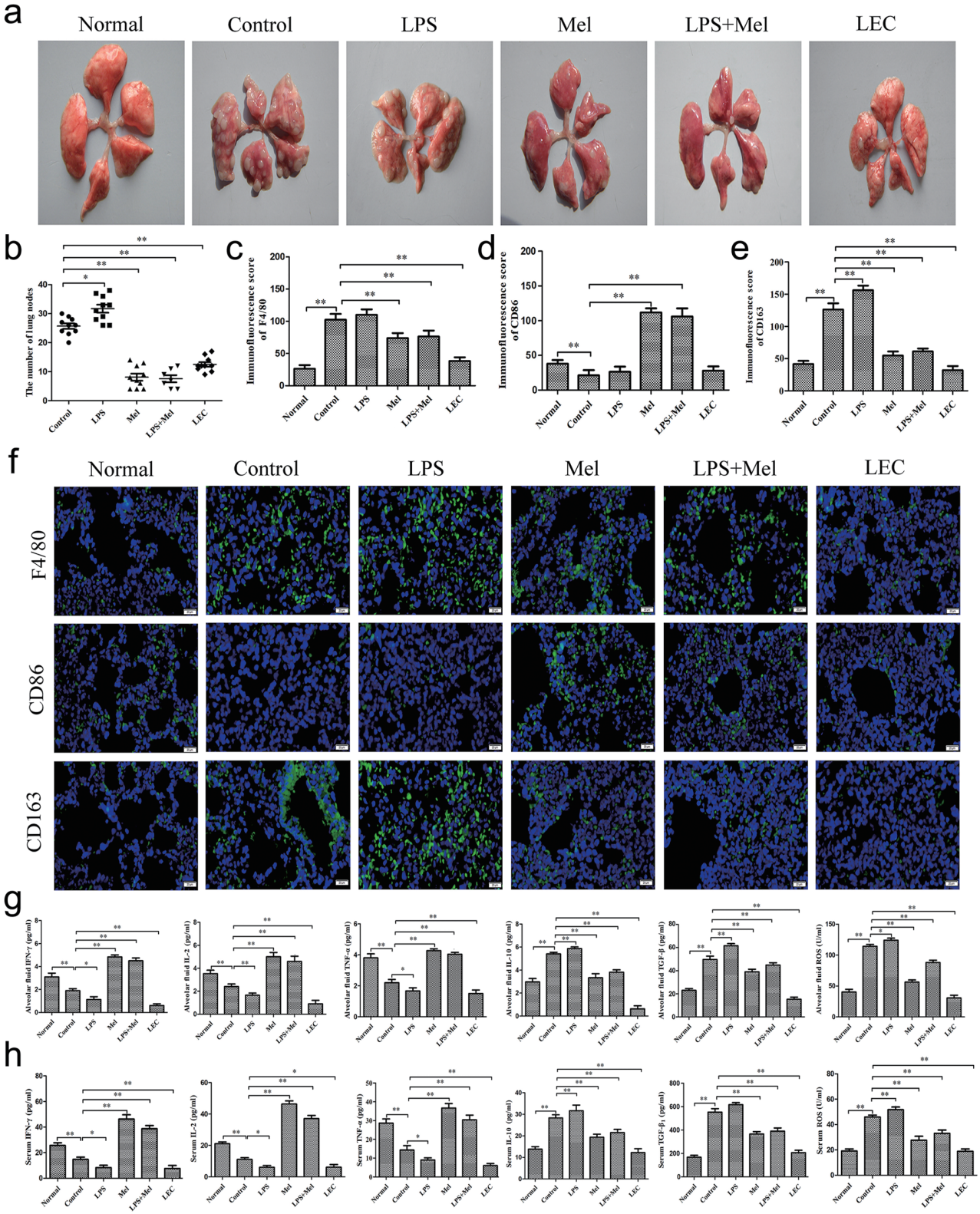

Fig. 3. Macrophages affect urethane-induced lung carcinogenesis. (a) The whole lung (n = 10). (b) The number of lung nodes (n = 10). (c), (d), (e) and (f) Surface markers of alveolar macrophages appeared by immunofluorescence (n = 5, 40×). (g) and (h) Serum and alveolar cytokine levels in urethane-induced lung cancer mice (n = 5). **P* < 0.05, ***P* < 0.01. Mel, Melatonin.

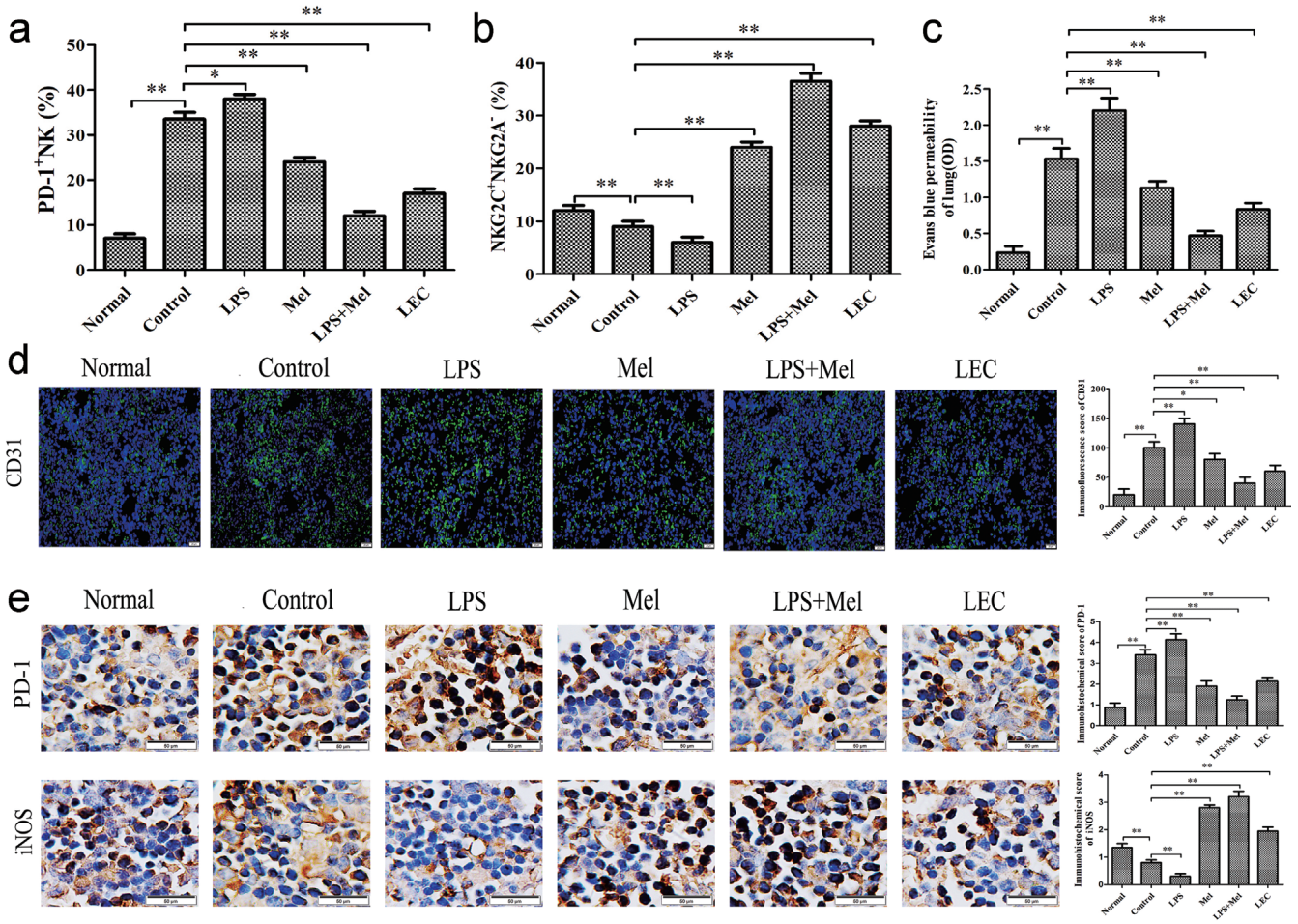

Fig. 4. Melatonin maintains immune function in urethane-induced lung carcinogenesis. (a) The percentage of spleen PD-1⁺ NK cells (n = 5). (b) The percentage of spleen memory NK cells (n = 5). (c) Lung tissue permeability were detected by Evans blue dye extra barrier experiment (n = 5). (d) Lung tissue CD31 expression detected by immunofluorescence (n = 5, 40×). (e) PD-1 and iNOS expression in lung tissues examined by immunohistochemistry (n = 5, 40×). **P* < 0.05, ***P* < 0.01. Mel, Melatonin.

Evans blue dye (Fig. 4c) and more CD31 immunofluorescence (Fig. 4d) in the control group compared with those normal mice, which indicates chronic inflammation and angiogenesis. Immunohistochemistry showed that lung tissue had more PD-1 and fewer iNOS in control groups compared with normal mice (Fig. 4e), suggesting an immunosuppressive microenvironment. LPS promoted this chronic inflammation, angiogenesis, and immunosuppressive microenvironment, while they were attenuated by melatonin alone or in combination with LPS and macrophage depletion induced by LEC (Fig. 4c–e).

Melatonin decreases M2 macrophages to promote LPS-induced tumor suppression and enhance tumor immunorejection of short-term LPS challenge in the LLC allograft model

To further investigate the influence of macrophages with different phenotypes in tumor progression, the allograft model of LLC tumor, synchronously, macrophages with M1 phenotype or M2 phenotype were intravenous injected into tumor-bearing mice separately. The M2 cell injection manifests the effect of promoting carcinoma, whereas the M1 cell injection manifests the effect on preventing carcinoma growth (Fig. 5a, b), indicating protumor and antitumor functions of macrophages, respectively. Unlike the lung cancer model induced by urethane, in the allograft model, melatonin (20 mg/kg) or macrophage depletion had an insignificant suppression on neoplasm growth, but LPS showed a significant suppressive effect on tumor progression (Fig. 5a, b). The tumor-suppressing effect of LPS was enhanced by melatonin, macrophage depletion, and injection of macrophages with M1 phenotype (Fig. 5a, b). In this model, the intratumor infiltration of Evans blue dye was increased by LPS and M1 cell injection alone or in combination but was deceased by LEC-induced macrophage depletion, melatonin, and M2 cell injection (Fig. 5c). The assay of intratumor macrophage phenotypes showed that the infiltrated macrophages expressed more surface CD163 than CD86 in the control group, indicating an M2 phenotype (Fig. 5d), which was reversed by LPS, M1 cell injection, and melatonin alone or in combination, but was promoted by M2 cell injection, indicating a dependence of LPS anti-tumor on M1 phenotype. Spleen NK cell analyses showed that PD-1⁺ NK cells increased and memory NK cells reduced in control mice compared with those of normal mice (Fig. 5e, f). However, contrary to the lung cancer model induced by urethane, the results of this model, indicate that the

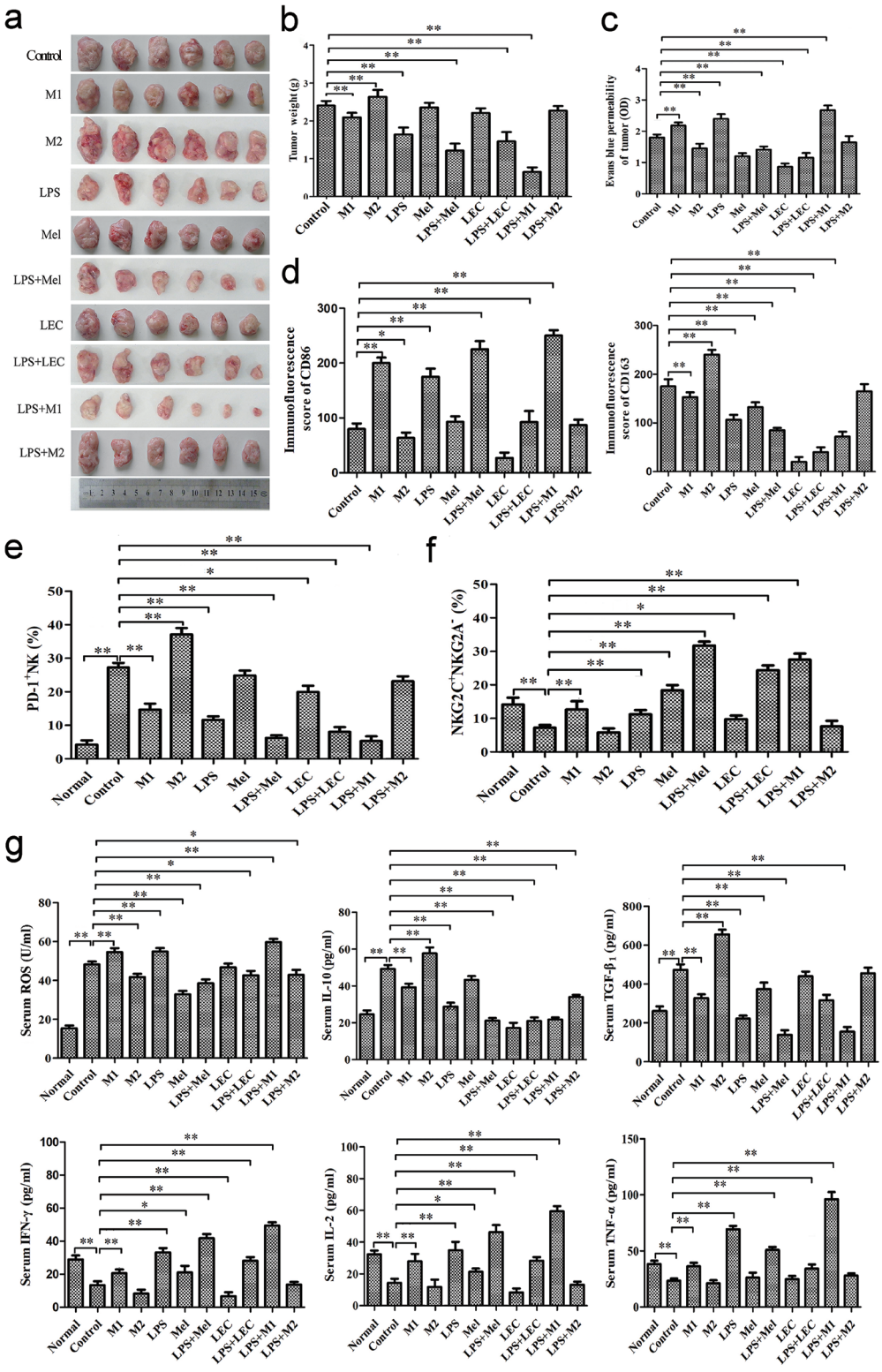

Fig. 5. Melatonin decreases M2 macrophage2 to promote the tumor-suppressing effect of short-term LPS challenge. (a) and (b) Tumor and tumor weight in an LLC tumor allotransplantation model (n = 6). (c) Lung tissue permeability were detected by Evans blue dye extra barrier experiment (n = 6). d) Peritoneal macrophage immunophenotypes detected by immunofluorescence (n = 6). (e) The percentage of spleen PD-1$^+$ NK cells (n = 6). (f) The percentage of spleen memory NK cells (n = 6). (g) Serum cytokine levels (n = 6). *P < 0.05, **P < 0.01. Mel: Melatonin. LEC, liposome-encapsulated clodronate; LLC, Lewis lung carcinoma; LPS, Lipopolysaccharide.

inhibitory effect of M2 phenotype on NK cell effect was also attenuated by LPS, while it was suppressed by M1 cell injection, melatonin, and LEC-induced macrophage depletion but was promoted by M2 cell injection (Fig. 5e, f). This result was proved by the levels of antitumor-related serum ROS, IFN-γ, IL-2 as well as TNF-α, and the levels of protumor-related serum IL-10 as well as TGF-β1 (Fig. 5g). The dependence of LPS anti-tumor on M1 phenotype was further proved in an LLC cell rechallenge immune experiment where the rechallenge control mice showed a smaller tumor than tumor-bearing normal mice, indicating tumor rejection in the tumor rechallenge mice (Fig. 6a). This tumor rejection was promoted by LPS and melatonin alone or in combination, and the combined effect of LPS and melatonin could be abrogated by LEC-induced macrophage depletion (Fig. 6a). The assay of peritoneal macrophage phenotype showed the correlation between tumor rejection and M1 cells indicated by surface CD86 and CD163 (Fig. 6b). Spleen NK cell assay showed the reduced PD-1$^+$ NK cells and the increased memory NK cells in rechallenge control mice compared to those of tumor-bearing normal mice (Fig. 6c, d), indicating a dependence of NK cell immunosurveillance on M1 cells. This result was also further confirmed by the levels of antitumor-related serum ROS, IFN-γ, IL-2 as well as TNF-α, and the levels of protumor-related serum IL-10 as well as TGF-β1 (Fig. 6e). These parameters could be shifted toward immunosurveillance by LPS and melatonin alone or in combination, and the combined effect of LPS and melatonin could be abrogated by LEC-induced macrophage depletion (Fig. 6b–e), revealing that macrophages play an indispensably important role in the secondary immunity and the immune memory.

Melatonin regulates macrophages by targeting the multi-protein network

We searched and collected 590 upregulated and 994 downregulated genes (P<0.05, LogFC ≤ −1.5), these genes are closely related to tumor-associated macrophages and 181 targets of melatonin regulating macrophages were selected by Docking scores (Fig. 7a). And 4 key genes (JAK2, STAT3, PIK3CA, and AKT1) were selected by the PPI network that was hub targets of melatonin regulating macrophages (Fig. 7b), A total of 136 potential targets have a docking score >6.0 (pKd/pKi) were screened out The analysis of GO enrichment elaborated that the potential targets of melatonin were mainly related to these biological processes, include “signal transduction”, “innate immune response”, “cell proliferation”, “protein phosphorylation” and “apoptotic process” (Fig. 8a). The analysis of KEGG enrichment elaborated that these potential targets were primally participated in the “Pathways in cancer”, “TNF signaling pathway” and “JAK/STAT signaling pathway” terms (Fig. 8b), which is consistent with our experimental results.

Discussion

Cancer may arise from alterations in different physiological processes and is refractory to cure due to unknown etiology and genetic heterogeneity. Based on self-healing ability, immunotherapy has been a great expectation against distinct types of cancer.[31–36] Current immunotherapies targeting different cellular checkpoint controllers emerged as having either innate or acquired resistance due to the immunosuppressive tumor environment, guiding the direction of developing cancer immunotherapy.[37] The object of cancer immunotherapy is to stimulate a long-lasting immunosurveillance, maintaining the antitumor immunity.[38] Macrophages, as the first-line immune responders in the innate immune system, are responsible for non-resolving inflammation in tumors. Understanding the roles of macrophages in the neoplastic immune response may help develop new immunotherapeutic strategies and enhance the response rate of immunotherapy.[39] Macrophages can manifest pro- or antitumorigenic effects, which depend on the immunophenotype. Recent studies have demonstrated that targeting TIMs can reverse the immunosuppressive tumor microenvironment and stimulate robust tumor-specific immune responses, which is consistent with the fact that immunosuppressive TIMs are abundant in the tumor microenvironment and are positively correlated with poor prognosis. Therefore, maintaining the antitumorigenic phenotype of macrophages rather than completely depleting TIMs represents a new cancer immunotherapy strategy.[40,41] LPS is an effective trigger of macrophage-mediated inflammation and is recognized as an effective antitumor drug in animal tumor models.[41] Its application, however, in human cancer treatment was not very successful due to LPS-induced tolerance, a state of altered responsiveness in macrophages, which results in poor tumor response and is a primary cause of secondary hospital infections. Previously, Novakovic *et al*. reported that β-glucan could reverse the epigenetic state of immunological tolerance induced by LPS to reduce overall sepsis mortality.[42] In this study, we first showed that the combination of LPS and melatonin could reduce macrophage polarization toward M2-like cells and therefore manifested a lasting antitumor effect, suggesting a novel effective strategy for reversing LPS tolerance.

Macrophages can be activated in response to different agents to become M1 and M2 macrophages and thus exert different functions. It is well known that LPS has the ability to polarize macrophages toward the M1 phenotype, which exerts the proinflammation and antitumor effects.[43] Consistent with these functions, in an animal model, LPS demonstrated a therapeutic effect on the transplanted tumor.[44] In small clinical trials, LPS also has the effect of alleviating symptoms and stabilizing the state of an illness in cancer patients.[45] However, LPS is responsible for the biological properties of bacterial endotoxins, which are potent inflammagens and result in fever, septic shock, toxic pneumonitis, and respiratory symptoms.[45] Although a cohort study suggested that long-term exposure to endotoxin was connected with a decreasing risk of lung cancer,[46,47] however, in clinical trials, LPS even pretreated with ibuprofen resulted in the unavoidable LPS-mediated clinical toxicities.[48] In addition, subsequent LPS-induced macrophage resistance after activation was also an obstacle that prevented durable tumor response to macrophages. Despite intense investigations of various epigenetic and genetic changes in tolerant macrophages for many years, a unifying mechanism that is responsible for LPS tolerance remains elusive.[49–51] It has been reported that the hyporesponsiveness of most LPS target genes in tolerant macrophages.[52] In the present study, Raw264.7 macrophages were induced to polarize to M1-like cells by a short-term LPS challenge but toward M2-like cells by a long-term LPS challenge, suggesting a critical process of LPS tolerance in macrophages.

Melatonin is a molecule with multifunction and has manifold physiological and pharmacological actions.[53] Melatonin generally plays a balancer in immunity and inflammation to check and balance.[54] Generally speaking, M1-like TIMs are regarded to be the regnant phenotype in early tumor stages, and M2-like TIMs are more regnant during the further process of cancer development.[44] Melatonin is an immunostimulator that drives an activated immunocyte state in favor of effectively clearing pathogens under normal or immunosuppressive conditions, while it acts as an immuno-

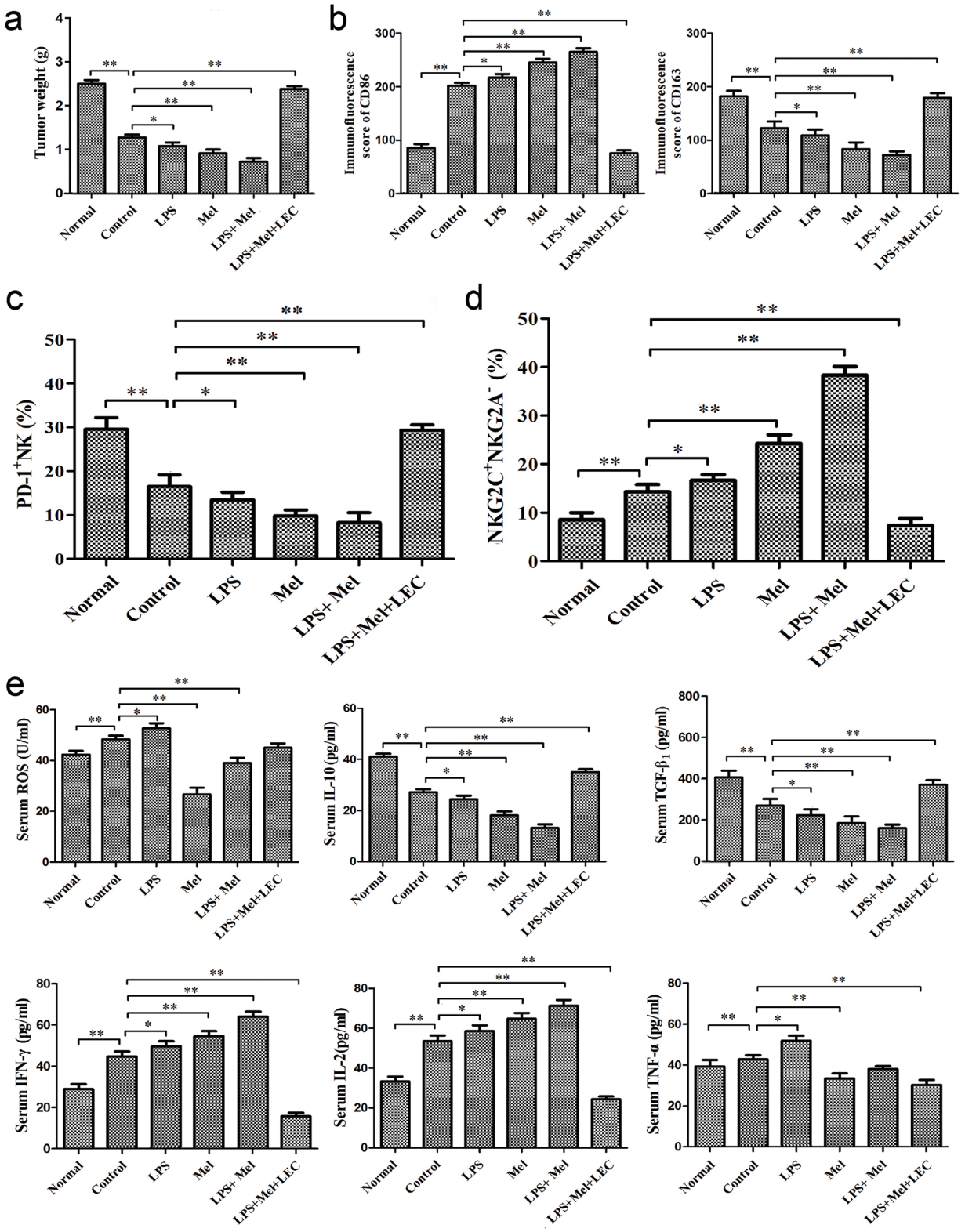

Fig. 6. Melatonin decreases M2 macrophages to keep tumor immune rejection. (a) Tumor weight in the rechallenge immune study. (n = 6). (b) Peritoneal macrophage immunophenotypes detected by immunofluorescence (n = 6, 40×). (c) The percentage of spleen PD-1$^+$ NK cells (n = 6). (d) The percentage of spleen memory NK cells (n = 6). (e) Serum cytokine levels (n = 6). *P < 0.05, **P < 0.01. Normal: tumor-bearing normal mice; Control: rechallenge control mice; Mel: Melatonin. LEC, liposome-encapsulated clodronate; LPS, Lipopolysaccharide.

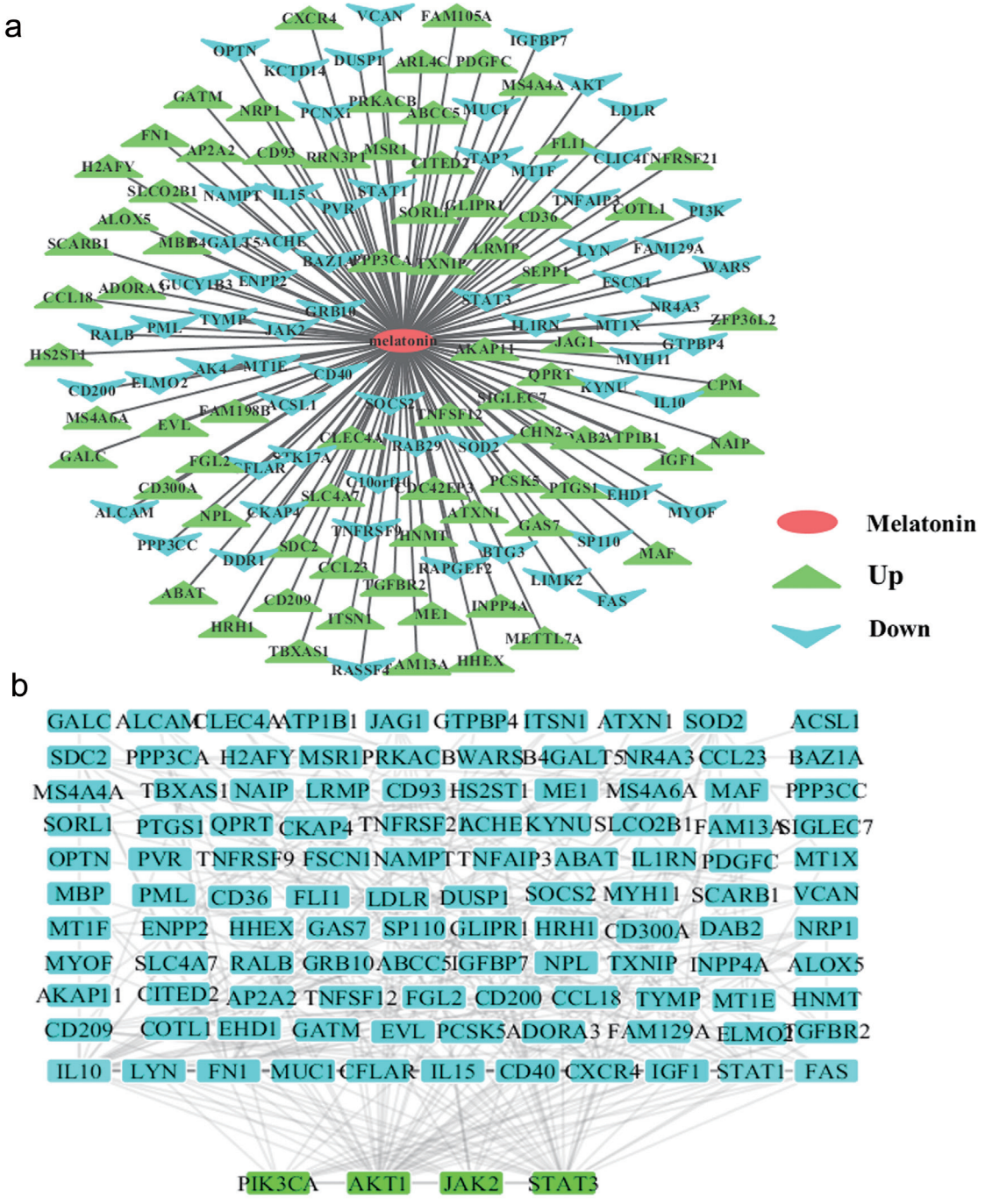

Fig. 7. The potential targets of melatonin in macrophages regulation. (a) The potential targets of melatonin in regulating macrophages. (b) The hub targets in PPI network. PPI, protein-protein interaction.

suppressor that urges immunocytes to enter an inactivated state that suppresses inflammatory reactions under excessively inflammatory conditions.[55,56] Several studies found that melatonin had an exciting potential to override the immunosuppressive tumor environment.[57] Various reports have shown that melatonin promotes 80% survival in lethal LPS-treated mice and significantly reduces the LPS-treated mortality in mice and rats by correcting the LPS-induced inflammatory imbalance with decreased levels of NO and lipid peroxidation.[58,59] In addition, a recent study demonstrated that melatonin could suppress indoleamine 2,3-dioxygenase-1 (IDO1) (a key immunomodulatory enzyme associated with cancer immune escape) to overcome tumor-mediated immunosuppression.[60,61] Based on these results, we believe that the combination of LPS and melatonin can maintain macrophage sensitivity to LPS and simultaneously limit excessive pathogenic stimuli to avoid a "macrophage exhaustion" phenotype. Consistent with our hypothesis, in this study, the combi-

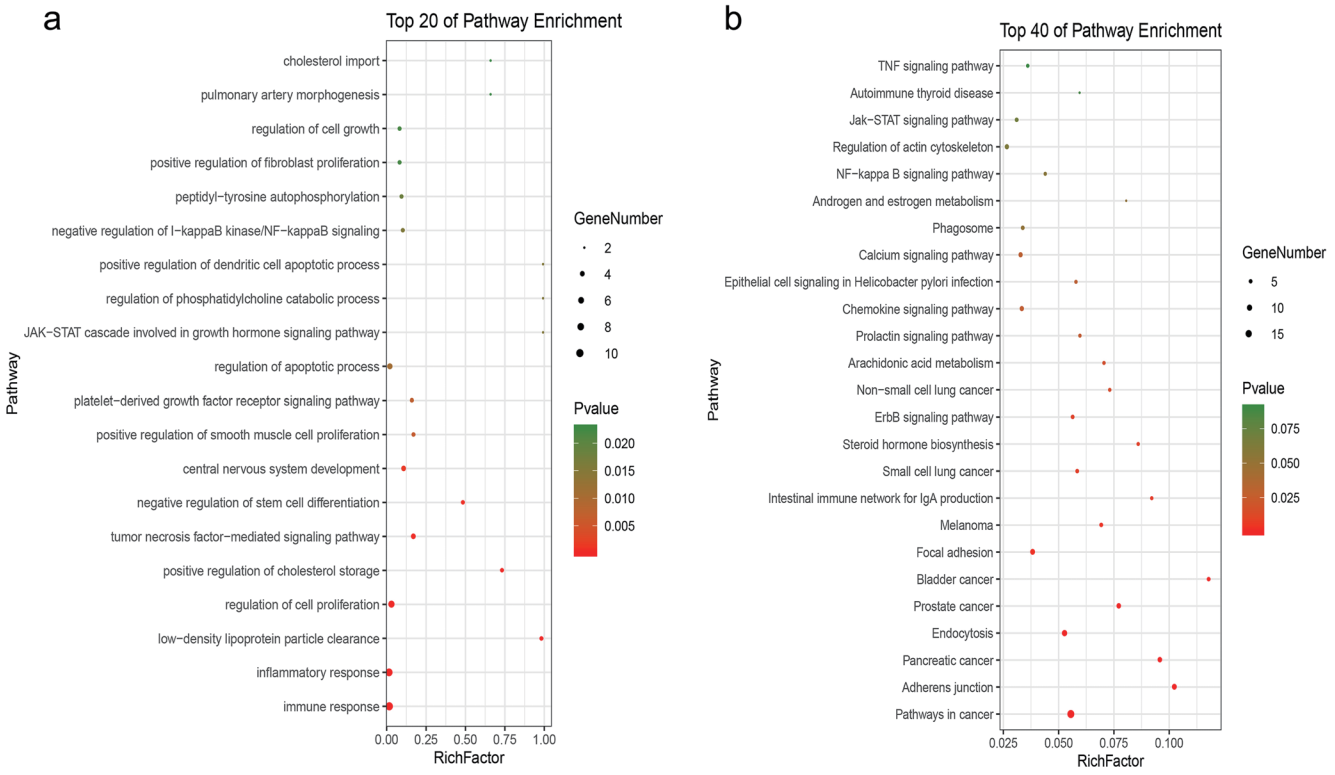

Fig. 8. GO and KEGG pathway performed by DAVID. (a) Top 20 pathway in GO enrichment analysis. (b) Top 40 pathway in KEGG enrichment analysis. DAVID, Database for Annotation, Visualization and Integrated Discovery; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

nation of LPS and melatonin resulted in optimal cancer prevention in a urethane-induced lung carcinogenic model and LLC allograft model without significant side effects. In addition, we found that if macrophages repeatedly challenged by LPS will have a high level of surface lipid rafts and JAK2/STAT3 activation, which prevented both M1-like polarization and immune responses, melatonin, however, could decrease surface lipid rafts and JAK2/STAT3 activation. The bioinformatic analysis illustrated that the potential targets of melatonin related to macrophages regulation were mainly involved in "inflammatory response", "signal transduction", "cell proliferation" "innate immune response" and "negative regulation of apoptotic process", indicating the function of melatonin acting on multi-target networks, such as "Jak-STAT signaling pathway", "Toll-like receptor signaling pathway" and "Chemokine signaling pathway", whereby melatonin induces M2 macrophage apoptosis to reverse LPS-stimulated immune tolerance. We used a pharmacological blocker to prove the importance of Jak-STAT signaling in the melatonin-maintained macrophage phenotype, and JAK2/STAT3 signal blockade prevented M2-like macrophages from producing PD-L1 and NK cells from expressing PD-1, whereas lipid raft depletion decreased M2-like macrophage JAK2/STAT3 signaling, indicating an association of M2 macrophage functions and the feedback loop of lipid rafts - JAK2/STAT3-PD-L1. Of course, it is just one of the important feedback loops.

Historically, bacterial therapy as oncolytic agents has been recognized for malignant brain tumors, which indicated that the survival time of patients with infections at the resection site of malignant glioma is prolonged, and cancer vaccines were assumed to be based on immunotoxins of bacterial origin.[62] It was previously reported that occupational exposure to endotoxin in organic material reduced the risk of lung cancer among workers employed ≤35 years but increased the risk of lung cancer among those employed >50 years, implying a cancer-promoting action of endotoxin tolerance.[63,64] In fact, LPS has been used for tumor destruction for many years, such as a report by Chicoine *et al*. that intratumor injection of LPS could cause the regression of subcutaneously implanted mouse glioblastoma multiforme in animal tumor models.[65] Goto S *et al*. also reported that intradermal LPS administration has a significant curative effect in three of five evaluable human cancer, suggesting that LPS is a potent antitumor agent.[66] However, only one trial of LPS used in human cancer therapy declared a poor tumor response and unbearable side effects. In addition, LPS inhalation can also produce both a systemic and a bronchial inflammatory response.[67,68] Therefore, it is necessary for LPS used as an antitumor agent to balance its inflammatory response and tolerance. In this study, melatonin induces M2 macrophage apoptosis and enhanced the anti-tumor effect of short-term LPS stimulation, providing a safe and effective strategy for the clinical application of LPS, and this therapeutic strategy is worth investigating.

Future directions

LPS as endotoxin, a cell wall component of gram-negative bacteria, was previously used for tumor destruction for many years, however, LPS use in human cancer therapy declared a poor tumor response with a cancer-promoting action of endotoxin tolerance, indicating LPS as either a potent antitumor agent or a cancer-promoting agent in an immune state dependent manner. Our study showed that the use of LPS combined with melatonin could promote tumor immu-

norejection, which suggests a correlation of M2 macrophage polarization with LPS-stimulated "macrophage exhaustion" and offers a novel therapeutic strategy for LPS preventing lung cancer. This strategy is to reeducate macrophages in the tumor microenvironment rather than to kill macrophages, which creates a harmony between system and immunity and is worth being explored in clinics.

Conclusions

In summary, our findings identify melatonin as a key maintainer of macrophage phenotypes to maintain system immune surveillance, which can induce LPS-stimulated M2 macrophage apoptosis and reverse tumor-promoting effect of the lasting LPS stimulation, and provide a novel strategy for immune-enhanced lung cancer prevention.

Supporting information

Supplementary material for this article is available at https://doi.org/10.14218/ERHM.2022.00014.

Supplementary File 1. ARRIVE checklist.

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Conflict of interest

The authors have no conflicts of interest related to this publication.

Author contributions

Study concept and design (ZG and GD), the performance of experiments (FG, YL, MZ, YN, WL, LS, HX, HL), analysis of data (GD, ZG, FG, YL, MZ), the contribution of reagents/materials/analysis tools (ZG and GD), manuscript writing and Drawing of the results (FG, ZG, and GD). All authors have made a significant contribution to this study and have approved the final manuscript.

Ethical Statement

This study was approved by the Animal Experimentation Ethics Committee of Henan University (permission number HUSAM 2016-288), and all procedures were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals and the Regulation of Animal Protection Committee.

Data sharing statement

All data generated or analyzed during this study are included in this article. No additional data are available.

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